[CANCER RESEARCH 45, 3460–3464, August 1985]

Investigation of Resistance to DNA Cross-Linking Agents in 9L Cell Lines with Different Sensitivities to Chloroethylnitrosoureas[1]

William J. Bodell,[2] Massimo Gerosa, Toshimitsu Aida, Mitchel S. Berger, and Mark L. Rosenblum[3]

Brain Tumor Research Center of the Department of Neurological Surgery, School of Medicine, University of California, San Francisco, California 94143

ABSTRACT

The 9L-2, 9L-7, and 9L-8 cell lines, derived from the 9L *in vivo* rat brain tumor, were treated with nitrosoureas that can alkylate and cross-link DNA and carbamoylate intracellular molecules to various extents. Compared to 9L cells, 9L-2 cells were very resistant to the cytotoxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea, and to 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-deoxyglucopyranose. The sensitivity of 9L-7 and 9L-8 cell lines to these drugs was intermediate between 9L and 9L-2. Treatment of 9L, 9L-2, 9L-7, and 9L-8 cell lines with 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea produced approximately the same level of cell kill. Compared to 9L cells, 9L-2 cells are 10-fold more resistant to the cytotoxic effects, 34-fold more resistant to the induction of sister chromatid exchanges, and have 40% fewer DNA interstrand cross-links caused by treatment with 3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(2-chloroethyl)-1-nitrosourea. In contrast, treatment of 9L and 9L-2 cells with 1-ethylnitrosourea produced approximately the same level of cell kill and induction of sister chromatid exchanges. Our results suggest that the resistance of 9L-2, 9L-7, and 9L-8 cells is related to DNA cross-linking and not to alkylation or carbamoylation.

We studied the effects of other agents that form DNA cross-links with structures different from those formed by treatment with chloroethylnitrosoureas (CENUs) in 9L and 9L-2 cells. In contrast to results obtained with CENUs, 9L-2 cells were 2-fold more sensitive to the cytotoxic effects, 2-fold more sensitive to the induction of sister chromatid exchanges, and had 3-fold more cross-links formed than 9L cells treated with nitrogen mustard. However, the amount of cell kill, number of sister chromatid exchanges induced, and the DNA cross-linking were the same for 9L and 9L-2 cells treated with *cis*-diamminedichloroplatinum(II).

Our results indicate that cellular resistance to CENUs is highly specific and that the mechanism of resistance does not allow cross-resistance with other DNA cross-linking agents. These and other results suggest that when DNA repair processes mediate cellular resistance to CENUs, other cross-linking agents will not be cross-resistant unless they form alkylation products that are affected by repair processes that mediate resistance to CENUs.

INTRODUCTION

Certain CENUs[4] are hydrolyzed intracellularly to reactive intermediates that alkylate DNA, form DNA interstrand cross-links, and carbamoylate cellular proteins. Because all of these reactions may cause cellular cytotoxicity, but with different efficiencies, it is necessary to establish which of these events is of primary importance to cell killing in sensitive cells and which processes are modified by cells resistant to the cytotoxic effects of these agents. Therefore, in this study we treated 9L cells and lines derived from the *in vivo* 9L rat brain tumor by BCNU treatment (1) with CENUs and other nitrosoureas with different capacities to alkylate DNA, cross-link DNA, and carbamoylate intracellular molecules (see Table 1). We measured cell kill, SCE induction, and DNA cross-link formation in order to obtain a better understanding of the molecular mechanisms of cell killing and cellular resistance to CENUs.

Preliminary investigations have shown that 9L-2 cells are resistant to the cytotoxic effects of BCNU and to the induction of SCEs compared to 9L-cells treated with the same concentration of BCNU (2). However, from these studies we could not determine if the observed cellular resistance was specific for CENUs, or whether 9L-2 cells were also resistant to other DNA-cross-linking agents. Schabel *et al.* (3, 4) have performed extensive studies on resistance and patterns of cross-resistance in L1210 and P388 cell lines. Schabel's results showed that L1210 and P388 cells resistant to BCNU were also resistant to other CENUs but retained the sensitivity of the parent line to phenylalanine mustard, cyclophosphamide, and *cis*-Pt (3, 4). Erickson *et al.* (5) have shown that human tumor cells resistant to BCNU are not cross-resistant to *cis*-Pt. Tew and Wang (6) have reported that Walker 256 rat carcinoma cells highly resistant to HN_2 and related derivatives are not cross-resistant with CENUs. These results suggest that cellular resistance to DNA cross-linking agents is highly specific. The experiments reported here were performed to determine whether 9L-2 cells resistant to BCNU are cross-resistant to other DNA cross-linking agents.

MATERIALS AND METHODS

Cell Lines. The 9L rat gliosarcoma is a well-established cell line (7). Sublines that are resistant to BCNU were produced by the following procedure. Fisher 344 rats bearing the intracerebral 9L tumor were treated with a single dose of BCNU, either 13.3 or 26.7 mg/kg. Twenty-four h after the dose was administered, rats were sacrificed, and tumors

[1] Supported in part by NIH Grants CA-13525 and CA-31882 and the Phi Beta Psi Sorority.

[2] To whom requests for reprints should be addressed, at the Brain Tumor Research Center, 783 HSW, University of California, San Francisco, CA 94143.

[3] Recipient of Teacher Investigator Award 1K-07NS 00604 NSPA from the National Institute of Neurological Communicative Disorders and Stroke.

Received 12/14/84; revised 4/23/85; accepted 5/7/85.

[4] The abbreviations used are: CENU, chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ENU, *N*-ethylnitrosourea; CHLZ, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose; BHCNU, 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; ACNU, 3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(2-chloroethyl)-nitrosourea; HN_2, nitrogen mustard; *cis*-Pt, *cis*-diamminedichloroplatinum(II); CFE, colony-forming efficiency; SCE, sister chromatid exchange.

Table 1
Functionality of agents used in this study

Agent	Alkylate	Cross-link	Carbamoylate
BCNU	+ (16, 28)[a]	+ (2, 12, 17)	+ (16, 28)
CHLZ	+ (29)	+ (12)	− (29)
BHCNU	− (14)	−	+ (14)
ACNU	+ (29)	+ (30)	− (29)
ENU	+ (15)	− (17)	+ (28)
HN_2	+ (21, 26)	+ (26, 31)	
cis-Pt	+ (32, 33)	+ (32, 33)	

[a] Numbers in parentheses, references for each agent.

were removed and disaggregated to single cells using an enzyme cocktail (8). Cells that survived treatment with 26.7 mg/kg were designated 9L-2, and cells that survived treatment with 13.3 mg/kg in 2 separate experiments were designated 9L-7 and 9L-8. The *in vivo* log kills observed were 3.36, 2.96, and 3.0 for 9L-2, 9L-7, and 9L-8, respectively. There was no discernible difference in morphology or karyotype between 9L and 9L-2 sublines.[5] The CFE for untreated 9L cells was 60 to 80%, and the plating efficiency of the 9L sublines was similar. 9L cells have an *in vitro* doubling time of approximately 18 to 19 h, and the 9L sublines showed similar growth rates. All 9L sublines have a base line SCE value of 11 to 12 SCEs/metaphase. Cells were passaged in culture and stored in liquid nitrogen. Cell lines were subcultured approximately twice a week.

Drugs. BCNU and *cis*-Pt were obtained from Bristol Laboratories (Syracuse, NY), CHLZ and BHCNU from the Drug Development Branch of the National Cancer Institute (Bethesda, MD), ACNU from Sankyo Drug Co. Ltd. (Tokyo, Japan), ENU from Ash Stevens Co. (Detroit, MI), and HN_2 from Sigma Chemical Co. (St. Louis, MO). All drugs were dissolved in solvents immediately before use. BCNU, ENU, CHLZ, and BHCNU were dissolved in absolute ethanol, and ACNU and HN_2 were dissolved in sterile water (pH 6.1); *cis*-Pt was dissolved in dimethyl sulfoxide and diluted with Hanks' balanced salt solution.

Drug Treatment and CFE Assay. Single-cell suspensions of 9L, 9L-2, 9L-7, and 9L-8 were obtained by incubation of confluent cultures with trypsin-EDTA; various numbers of cells (50 to 1×10^4) were plated into 60-mm Petri dishes or 25-sq cm flasks with heavily irradiated 9L feeder cells in Eagles' minimum essential medium containing 10% fetal calf serum and gentamycin (50 μg/ml). After 24 h of incubation, cells were treated with various concentrations of the drugs for either 1 h followed by medium replacement or without a medium change ("continuous treatment"). Cultures were then incubated in a 5% CO_2:95% air atmosphere for 10 to 14 days. After being fixed with methanol:acetic acid and stained with crystal violet, colonies containing 50 or more cells were scored. Surviving fraction was calculated as the ratio of the CFEs of treated and untreated cells. There was a direct relationship between number of cells plated and colonies formed for the data obtained with the CFE assay.

SCE Assay. Cells (1.0 to 1.5×10^6) were seeded into 75-sq cm flasks. The following day, cells were treated with various concentrations of the drugs for 1 h. After treatment, medium was removed and replaced with medium containing bromodeoxyuridine, 10 μmol/liter, after which cells were incubated for 2 replication cycles (approximately 28 h). Mitotic cells were accumulated by treatment with colcemid, 0.04 μg/ml, for 2 h. Flasks were shaken to dislodge the mitotic cells, medium was poured off, and mitotic cells were collected by centrifugation (1000 rpm for 5 min). The pellet was treated with 2.0 ml of 0.05 M KCl for 8 to 10 min, fixed twice with glacial acetic acid and methanol (1:3), and metaphase chromosomes were spread on glass microscope slides. The method of Perry and Wolff (9) was used for differential staining of sister chromatids. For each experiment, the frequency of SCEs was determined in 25 metaphase cells.

Alkaline Elution Assay. Cells (0.5 to 1×10^6) were seeded into 100-mm dishes and grown for 2 to 3 days in medium containing either [^{14}C]thymidine, 0.01 μCi/ml (60 mCi/mmol) or [^{3}H]thymidine, 0.1 μCi/ml (82.7 Ci/mmol). One h before drug treatment, medium containing labeled compounds was removed and replaced with fresh medium. ^{14}C-labeled cells were treated with various concentrations of ACNU, *cis*-Pt, or HN_2 for 1 h at 37°C. After treatment, the drug-containing medium was removed. The 1-h treatment period is sufficient for formation of the HN_2 DNA cross-links; therefore, after the treatment period, HN_2-treated cells were placed in cold Hanks' balanced salt solution and collected for alkaline elution. The ACNU- and *cis*-Pt-treated cells were incubated in drug-free medium at 37°C for an additional 6 h to allow for the formation of DNA interstrand cross-links and then collected for alkaline elution. ^{3}H-labeled cells were irradiated with 300 rads, and the ^{14}C-labeled cells with 600 rads of X-rays (General Electric Maxitron 300). Comparison of the elution pattern of ^{3}H-labeled cells with control ^{14}C-labeled cells provides an internal control to assure that DNA is eluting off the filter as a function of molecular weight. The elution procedure used as a modification of that described by Kohn *et al.* (10), and a detailed procedure has been described (2). The general procedure is as follows. Cells were collected on the filters, lysed, and treated with proteinase K for 1 h. DNA is eluted from the filters overnight. Results were calculated as the fraction of [^{14}C]DNA and [^{3}H]DNA retained on the filter at each fraction. The cross-linking index was calculated using the formula of Ewig and Kohn (11)

$$\left[\frac{(1 - R_0)}{(1 - R_1)}\right]^{1/2} - 1$$

where R_0 and R_1 are the relative retention for untreated and treated cells, respectively. Relative retention was defined as the fraction of the [^{14}C]DNA remaining on the filter when 50% of the [^{3}H]DNA remained on the filter.

RESULTS

Survival curves for 9L, 9L-2, 9L-7, and 9L-8 cells treated with BCNU and CHLZ are shown in Chart 1, *A* and *B*. At a concentration of 30 μM, BCNU and CHLZ produced a 2-log cell kill in 9L cells. In contrast, treatment of 9L-2 cells with 70 μM BCNU produced only a 1-log cell kill, and treatment with 70 μM CHLZ produced very little cell kill. Treatment with BCNU produced the same intermediate levels of cell kill in 9L-7 and 9L-8 cell lines (Chart 1*A*). For CHLZ, the levels of cell kill for 9L-7 and 9L-8 cells were intermediate between that for 9L and 9L-2; 9L-7 cells were somewhat more resistant than were 9L-8 cells (Chart 1*B*). All cell lines were equally susceptible to the cytotoxic effects of BHCNU, however (Chart 1*C*).

Survival curves for 9L and 9L-2 cells treated with ACNU are shown in Chart 2. 9L-2 cells were very resistant to the cytotoxic effects of ACNU; at a 1 log of cell kill, 9L-2 cells were 10-fold more resistant to the cytotoxic effects of ACNU than were 9L cells. ACNU induced SCEs in 9L cells; the dose-response curve is linear with a slope of 15.8 SCEs/metaphase/μM ACNU (all slopes were determined by linear regression analysis) (Chart 2). Treatment of 9L-2 cells with ACNU over the same dose range induced very few SCEs; the slope of the SCE dose-response curve is 0.46 SCEs/metaphase/μM ACNU. Therefore, calculated as the ratio of the slopes for induction of SCEs, 9L-2 cells were 34-fold more resistant to the induction of SCEs caused by treatment with ACNU than were 9L-cells. Comparison of the cross-linking index for 9L and 9L-2 cells treated with ACNU shows that there were on the average 40% fewer proteinase K-resistant DNA interstrand cross-links formed in 9L-2 than in 9L cells treated with ACNU (Table 2).

9L and 9L-2 cells were equally sensitive to the cytotoxic effects

[5] J. Trent, personal communication, 1985.

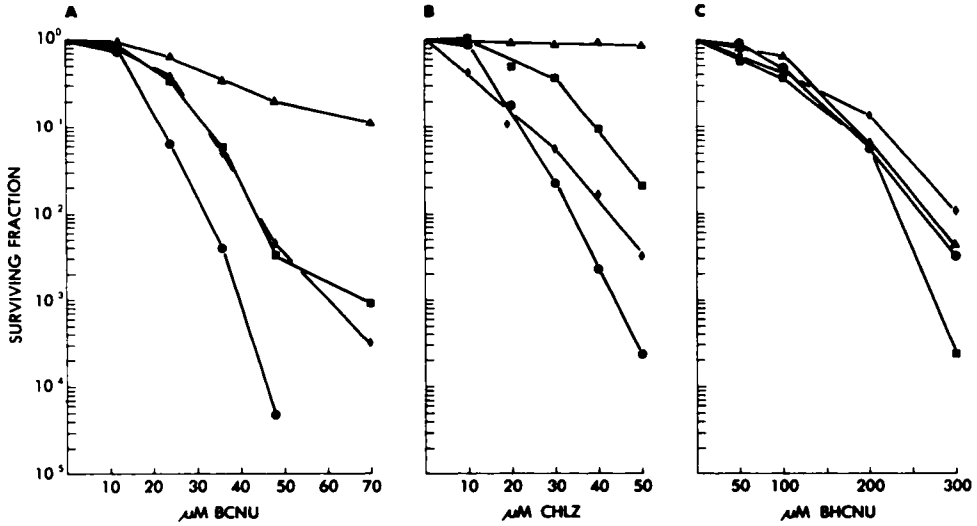

Chart 1. The survival of 9L (●), 9L-2 (▲), 9L-7 (■), or 9L-8 (◆) cells after continuous treatment with BCNU (*A*), CHLZ (*B*), or BHCNU (*C*).

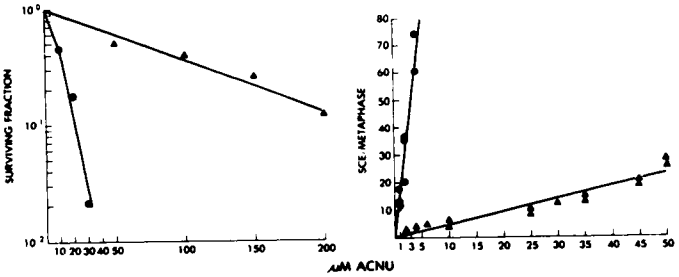

Chart 2. The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with ACNU. The number of SCEs induced is calculated by subtracting the number of SCEs induced by 10 μM bromodeoxyuridine alone.

Table 2

Cross-linking index for 9L and 9L-2 cells treated with ACNU, HN_2, and cis-Pt

Drug concentration (μM)	Cross-linking index × 1000	
	9L	9L-2
ACNU		
50	96 ± 10[a]	68 ± 3
100	275 ± 8	128 ± 18
HN_2		
0.5	72 ± 8	210 ± 25
1	152 ± 19	472 ± 79
2	333 ± 53	810 ± 99
cis-Pt		
30	228 ± 85	285 ± 42
60	454 ± 155	436 ± 35
90	572 ± 147	646 ± 54

[a] Mean ± SD.

of ENU (Chart 3). Treatment of both cell lines with 5 mM ENU produced a 1-log cell kill and induced a similar number of SCEs in both cell lines (Chart 3). Slopes of the SCE dose-response curves are 0.115 SCEs/metaphase/μM ENU for 9L cells and 0.086 SCEs/metaphase/μM ENU for 9L-2 cells.

Survival curves for 9L and 9L-2 cells treated with HN_2 are shown in Chart 4. In contrast to results found for treatment with CENUs, 9L cells were approximately 2-fold more resistant to the cytotoxic effects of HN_2 than were 9L-2 cells. Slopes of the SCE dose-response curves are 308 SCEs/metaphase/μM HN_2 for 9L cells and 660 SCEs/metaphase/μM HN_2 for 9L-2 cells. Calculated as the ratio of the slopes, 9L cells are 2-fold more resistant to the induction of SCEs than are 9L-2 cells treated with HN_2. Treatment of either 9L or 9L-2 cells with HN_2 for 1 h produced high levels of proteinase K-resistant DNA interstrand cross-links (Table 2). The extent of DNA cross-linking in 9L-2 cells was approximately 3-fold higher than for 9L cells treated with equimolar concentrations of HN_2. This result is in good agreement with both the differences in the number of SCEs induced and the differences in cell kill in 9L and 9L-2 cells treated with HN_2.

The level of cell kill and the number of SCEs induced is the same in 9L and 9L-2 cells treated with equimolar concentrations of *cis*-Pt (Chart 5). Slopes of the SCE dose-response curves are 11.1 SCEs/metaphase/μM of *cis*-Pt for 9L and 9.1 SCEs/metaphase/μM for 9L-2 cells. The values of the cross-linking index for 9L and 9L-2 cells treated with equimolar concentrations of *cis*-Pt are also similar (Table 2).

DISCUSSION

The cytotoxic response of 9L, 9L-2, 9L-7, and 9L-8 cells treated with BCNU and CHLZ have been measured. In contrast to 9L, 9L-2 cells were very resistant to the cytotoxic effects of these nitrosoureas, and 9L-7 and 9L-8 cells had intermediate responses. The dose-response curve for 9L cells treated with ACNU was very similar to 9L cells treated with BCNU and CHLZ. 9L-2 cells were more sensitive to treatment with BCNU than they were to ACNU or CHLZ, however, suggesting that carbamoylation of proteins by BCNU may either decrease the degree of cellular resistance or lead to additional cytotoxicity by itself. Similar results have been obtained by Erickson *et al.* (12) with IMR-90 and VA-13 cells treated with these nitrosoureas.

A good correlation between SCE induction and cellular sensitivity to BCNU has been found (2). In agreement with this, 9L-7, and 9L-8 cells have been shown to have an intermediate response to SCE induction caused by BCNU and CHLZ (13). Compared to 9L cells, 9L-2 cells were 34-fold more resistant to SCE induction and 10-fold more resistant to cell killing by ACNU treatment. These results suggest that the biochemical events that lead to SCE induction and cell killing are related.

Treatment of cells with certain CENUs results in DNA alkylation, formation of DNA interstrand cross-links, and carbamoylation of cellular proteins (Table 1). Biochemical modification of any

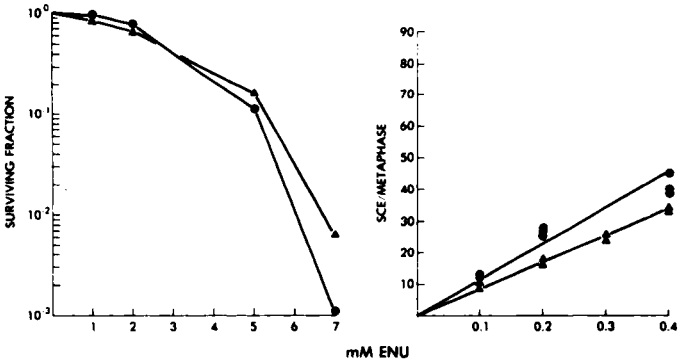

Chart 3. The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with ENU.

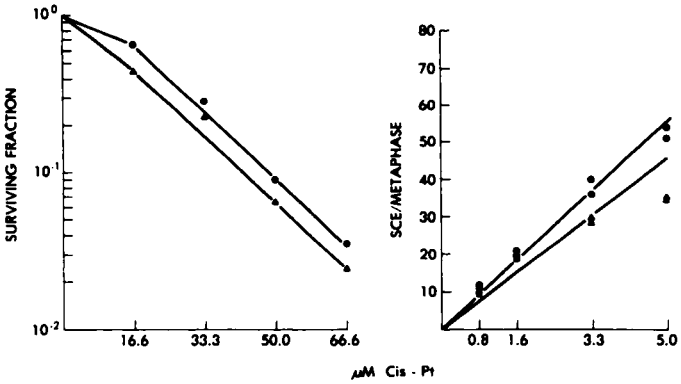

Chart 5. The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with *cis*-Pt.

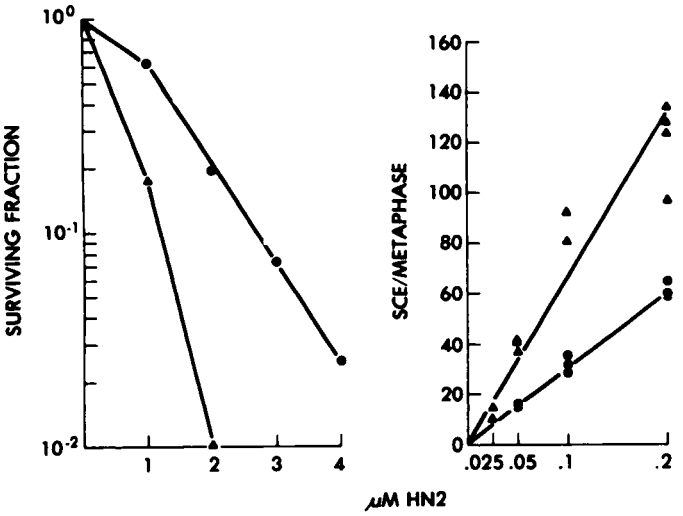

Chart 4. The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with HN_2.

of these processes could result in cellular resistance. To obtain a better understanding of how the resistance of 9L-2 cells to the cytotoxic effects of these agents is expressed, we investigated the effects of other nitrosoureas that were deficient for either alkylation, cross-linking, or carbamoylation.

9L, 9L-2, 9L-7, 9L-8 cells were treated with BHCNU, which does not alkylate or cross-link but does carbamoylate (14). The cytotoxic response of the 4 cell lines was similar indicating that all were equally susceptible to cytotoxicity caused by carbamoylation. 9L and 9L-2 cells were treated with ENU, a nitrosourea that alkylates (15) and presumably carbamoylates (16) but does not form DNA interstrand cross-links (17). 9L and 9L-2 cells were equally susceptible to both the cytotoxic effects of ENU and to the induction of SCEs. The results obtained with ENU and BHCNU suggest that cellular resistance to CENUs is associated with the DNA cross-linking properties of the CENUs.

Results obtained with alkaline elution show that the number of DNA cross-links caused by ACNU treatment in 9L-2 cells was 40% lower than in 9L. These results are similar to our finding for BCNU treatment of 9L and 9L-2 cells (2) and further support our conclusion that the lower number of DNA cross-links in 9L-2 cells is responsible in part for the observed cellular resistance to CENUs. Recent studies have also shown that cellular resistance of human tumor cells is associated with a reduced frequency of DNA interstrand cross-links caused by CENUs (18, 19).

Additional evidence that DNA cross-linking is an important factor in the cytotoxicity of CENUs was obtained by comparing the concentration of ENU or ACNU required to produce a 1-log cell kill. The cross-linking drug ACNU was 250-fold more cytotoxic than the noncross-linking ENU. Similar observations have been made by comparing methylnitrosourea and CENUs (20) and sulfur mustard with its noncross-linking analogue (21, 22). Thus, although the DNA interstrand cross-links formed by CENUs represent only 1% of the total alkylation products (23), they very effectively cause the events that lead to cell death. The nonalkylating nitrosourea BHCNU was also cytotoxic to cells, however, which indicates that additional mechanisms have to be considered in the evaluation of the mechanism of cytotoxicity of nitrosoureas.

The results obtained with CENUs indicate that cellular resistance in 9L-2 cells is related to reduced levels of DNA cross-links. We determined whether this resistance was specific for CENUs or was part of a general pattern of resistance to DNA cross-linking agents. The cytotoxicity, number of SCEs induced, and number of DNA cross-links formed in 9L and 9L-2 cells treated with *cis*-Pt were very similar. Therefore, 9L-2 cells were not cross-resistant to *cis*-Pt. The results obtained with HN_2, however, were in sharp contrast to results obtained with CENUs. 9L cells were 2- to 3-fold more resistant to the cytotoxic effects, induction of SCEs, and formation of DNA cross-links than were 9L-2 cells. Recent studies have shown that 9L cells contain approximately twice as much reduced glutathione as do 9L-2 cells,[6] which correlates with their increased resistance to NH_2 (24). These results show clearly that the cellular resistance to CENUs is highly specific and does not result in cross-resistance to other bifunctional agents. Similar results have been obtained by Schabel *et al.* (3, 4) using L1210 and P388 cell lines resistant to BCNU.

The reduced formation of DNA interstrand cross-links in 9L-2 cells (2) and resistant human cells treated with CENUs appears to be the result at least in part of increased repair of O^6-2-chloroethyl guanine (12, 19); the initial alkylation product leading to formation of the CENU induced DNA interstrand cross-link 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]ethane (25). The cross-link formed by HN_2 is bis(2-guanin-7-ylethyl)methylamine (26), which is different in structure from the cross-link caused by CENUs (1, 23, 25). Work with semipurified mammalian enzymes

[6] M. T. Smith, personal communication, 1985.

has shown that recognition and removal of alkylation products is very specific (27). Therefore, the results of this study suggest that when resistance to a particular chemotherapeutic agent is mediated by a specific repair process(es), other cross-linking agents will not be cross-resistant unless they form DNA alkylation products that are also substrates for that particular repair process(es). When cellular resistance is mediated by drug uptake, glutathione levels, glutathione-*S*-transferase, or gene amplification, however, different patterns of sensitivity and resistance probably will be observed.

ACKNOWLEDGMENTS

We thank Jeffrey Trent for performing the karyotype analysis and H. T. R. Rupniak for performing some of the initial cell survival studies.

REFERENCES

1. Rosenblum, M. L., Gerosa, M. A., Bodell, W. J., and Talcott, R. E. Tumor cell resistance. Prog. Exp. Tumor Res., *27:* 191–214, 1984.
2. Bodell, W. J., Rupniak, H. T. R., Rasmussen, J., Morgan, W. F., and Rosenblum, M. L.: Reduced level of DNA cross-links and sister chromatid exchanges in 1,3-bis(2-chloroethyl)1-nitrosourea resistant rat brain tumor cells. Cancer Res., *44:* 3763–3767, 1984.
3. Schabel, F. M., Tracher, M. W., Laster, W. R., Wheeler, G. P., and Witt, M. H. Patterns of resistance and therapeutic synergism among alkylating agents. Fundamentals in cancer chemotherapy antibiotics. Chemotherapy, *23:* 200–215, 1978.
4. Schabel, F. M., Skipper, H. E., Trader, M. W., Laster, W. R., Corbett, T. H., and Griswald, D. P. Concepts for controlling drug resistant cells. *In* H. T. Mouridsen and T. Palshot (eds.), Breast Cancer: Experimental and Clinical Aspects, pp. 199–211. Elmsford, NY: Pergamon Press, Inc., 1980.
5. Erickson, L. C., Zwelling, L. A., Ducore, J. M., Sharkey, N. A., and Kohn, K. W. Differential cytotoxicity and DNA cross-linking in normal and transformed human fibroblasts treated with *cis*-diamminedichloroplatinum(II). Cancer Res., *41:* 2791–2794, 1981.
6. Tew, K. D., and Wang, A. L. Selective cytotoxicity of haloethylnitrosoureas in a carcinoma cell line resistant to bifunctional nitrogen mustards. Mol. Pharmacol., *21:* 729–738, 1982.
7. Barker, M., Hoshino, T., Gurcay, O., Wilson, C. B., Nielsen, S. L., Downie, R., and Eliason, J. Development of an animal brain tumor model and its response to therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res., *33:* 976–986, 1973.
8. Rosenblum, M. L., Vasquez, D. A., Hoshino, T., and Wilson, C. B. Development of a clonogenic cell assay for human brain tumors. Cancer (Phila.), *41:* 2305–2314, 1978.
9. Perry, P., and Wolff, S. New Giemsa method for differential staining of sister chromatids. Nature (Lond.), *251:* 156–158, 1974.
10. Kohn, K. W., Ewig, R. A., Erickson, L. C., and Zwelling, L. A. DNA repair. *In:* E. C. Friedberg and P. C. Hanawalt (eds.), A Laboratory Manual of Research Procedures, pp. 279–301. New York: Marcel Dekker, Inc., 1981.
11. Ewig, R. A. G., and Kohn, K. W. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. Cancer Res., *38:* 3197–3203, 1978.
12. Erickson, L. C., Bradley, M. O., Ducore, J. M., Ewig, R. A. G., and Kohn, K. W. DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. Proc. Natl. Acad. Sci. USA, *77:* 467–471, 1980.
13. Tofilon, P. J., Gerosa, M. A., Rosenblum, M. L., Bodell, W.J., and Deen, D. F. Prediction of the relative *in vitro* sensitivity of 9L rat brain tumor cells to nitrosoureas by the sister chromatid exchange assay. Life Sci., *35:* 1611–1614, 1984.
14. Johnston, T. P., Wheeler, G. P., McCaleb, G. S., Bowden, B. J., and Montgomery, J. A. Nitrosoureas as potential radiosynergists. *In:* B. Serrou, P. S. Schein, and J. L. Imbach (eds.), Nitrosoureas in Cancer Treatment, pp. 139–151. Amsterdam: Elsevier/North-Holland Biomedical Press, 1981.
15. Singer, B., Bodell, W. J., Cleaver, J. E., Thomas, G. H., Rajewsky, M. F., and Thon, W. Oxygens in DNA are main targets for ethylnitrosourea in normal and xeroderma pigmentosum fibroblast and fetal rat brain cells. Nature (Lond.), *276:* 85–88, 1978.
16. Wheeler, G. P. A review on the mechanism of action of nitrosoureas. ACS (Am. Chem. Soc.), Symp. Ser., *30:* 87–119, 1976.
17. Lown, J. W., McLaughlin, L. W., and Chang, Y. M. Mechanisms of actions of 2-haloethylnitrosoureas on DNA and its relation to their antileukemic properties. Bioorg. Chem., *7:* 97–110, 1978.
18. Erickson, L. C., Laurent, G., Sharkey, N. A., and Kohn, K. W. DNA crosslinking, and monoadduct repair in nitrosourea-treated human tumor cells. Nature (Lond.), *288:* 727–729, 1980.
19. Zlotogorski, C., and Erickson, L. C. Pretreatment of normal human fibroblasts and human colon carcinoma cells with MNNG allows chloroethylnitrosourea to produce DNA interstrand crosslinks not observed in cell treated with chloroethylnitrosourea alone. Carcinogenesis (Lond.), *4:* 759–763, 1983.
20. Bradley, M. O., Sharkey, N. A., Kohn, K. W., and Layard, M. W. Mutagenicity and cytotoxicity of various nitrosoureas in V-79 Chinese hamster cells. Cancer Res., *40:* 2719–2725, 1980.
21. Brookes, P., and Lawley, P. D. The reaction of mono and difunctional alkylating agents with nucleic acids. Biochem. J., *80:* 496–503, 1961.
22. Roberts, J. J., Brent, T. P., and Crathorn, A. R. Evidence for the inactivation and repair of the mammalian DNA template after alkylation by mustard gas and half mustard gas. Eur. J. Cancer, *7:* 515–525, 1971.
23. Ludlum, D. B., Tong, W. P., Erickson, L. C., and Kohn, K. W. Comparison of DNA alkylation by ^{14}C-CCNU in L1210 cells and *in vitro.* Proc. Am. Assoc. Cancer Res., *25:* 290, 1984.
24. Suzukake, K., Petro, B. J., and Vistica, D. T. Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. Biochem. Pharmacol., *31:* 121–124, 1982.
25. Tong, W. P., Kirk, M. C., and Ludlum, D. B. Formation of the cross-link 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]ethane in DNA treated with *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea. Cancer Res., *42:* 3102–3105, 1982.
26. Brookes, P., and Lawley, P. D. The alkylation of guanosine and guanylic acid. J. Chem. Soc. (Lond.), 3923–3028, 1961.
27. Lindahl, T. DNA repair enzymes. Annu. Rev. Biochem., *51:* 61–87, 1982.
28. Wheeler, G. P., Bowdon, B. J., Grimsley, J. A., and Lloyd, H. H. Interrelationships of some chemical, physicochemical, and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. Cancer Res., *34:* 194–200, 1974.
29. Heal, J. M., Fox, P., and Schein, P. S. A structure activity study of seven new water soluble nitrosoureas. Biochem. Pharmacol., *28:* 1301–1306, 1979.
30. Fujimoto, S., Ozawa, M., and Sakurai, Y. Hypothetical mechanism of therapeutic synergism induced by the combination of 6-thioguanine and 3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(2-chloroethyl)-1-nitrosourea hydrochloride. Cancer Res., *42:* 4079–4085, 1982.
31. Murnane, J. E., and Byfield, J. C. Irrepairable DNA crosslinks and mammalian cell lethality with bifunctional alkylating agents. Chem.-Biol. Interact., *38:* 75–86, 1981.
32. Roberts, J. J., and Friedlos, F. The frequency of interstrand crosslinks in DNA following reaction of *cis*-diamminedichlorplatinum (II) with cells in culture or DNA *in vitro*: stability of DNA crosslinks and their repair. Chem.-Biol. Interact., *39:* 181–189, 1982.
33. Zwelling, L. A., Bradley, M. O., Sharkey, N. A., Anderson, T., and Kohn, K. W. Mutagenicity cytotoxicity and DNA crosslinking in V-79 Chinese hamster cells treated with *cis* and *trans*-Pt (II) diamminedichloride. Mutat. Res., *67:* 271–280, 1979.